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Antiviral Activities of *Capsicum annuum* Methanolic Extract against Herpes Simplex Virus 1 and 2

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ABSTRACT

This study was conducted to quantify the antioxidant and anti-viral activities of *Capsicum annuum* methanolic extracts. The extract of *Capsicum annuum* was prepared by maceration method and the total polyphenolic and flavonoids contents were determined using a spectrophotometric method. The antiradical scavenging property and the maximum nontoxic concentration of the extract was determined using DPPH assay and MTT method, respectively. Serial concentrations of the extract not exceed the maximum nontoxic concentration were used against Vero cells for anti-HSV-1 and ant-HSV-2 effects by plaque assay. The results of the phytochemical analysis showed that the extract has high polyphenolic content while has relative low flavonoids contents. In the antiradical scavenging property test the extract showed at 64.1 µg/ml 50% DPPH radical scavenging activity and in the MTT assay the extract has relatively low cytotoxicity where the IC_{50} value of extract against Vero cells was 1078.69 µg/ml. Furthermore, the extract exhibited a considerable anti-HSV-1 and anti-HSV-2 activities at the concentration of 25 µg/ml. In conclusion, our findings indicate that the methanolic extract of *Capsicum annuum* could be used in combination with standard drug to treat herpes infection.

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TH, MM, MD and SQ designed and performed the experiments. TH and MM wrote the paper.

Key words
***Capsicum annuum*, Herpes simplex virus type-1, Herpes simplex virus type-2, Antioxidant, Antiviral.**

INTRODUCTION

Natural products and medicinal herbs have demonstrated to be a principle source of forefront molecules and many plant extracts as crude or an active ingredient with antiviral activity have been documented (Alvarez *et al*., 2015; Visintini Jaime *et al*., 2013). *Capsicum* is a genus belongs to family *Solanaceae* or nightshades that are an economically important family of flowering plants. Capsicum is highly rich in vitamins A, B, C and E and also contains many minerals such as molybdenum, potassium and manganese. According to Levy *et al*. (1995) and Szolcsanyi (2004), liquid chromatography–mass spectrometry analysis revealed the presence of violaxanthin ($C_{40}H_{56}O_4$), capsaicinoids ($C_{18}H_{27}O_3N$), lutein ($C_{40}H_{56}O2$), β-cryptoxanthin ($C_{40}H_{56}O$) and β-carotene ($C_{40}H_{56}$) in *Capsicum* fruits. Furthermore, Liu *et al*. (2013) found three capsaicinoids like capsaicin, dihydrocapsaicin and nordihydrocapsaicin in extracts of *Capsicum annuum*. In folk medicine, *Capsicum* is used to relief neuralgia, rheumatic disorders and non-allergic rhinitis and as irritant in lumbago. Moreover, *Capsicum* has been shown to have an antimicrobial activity inhibiting the growth of gram positive and gram negative bacteria such as *Staphyllococcus* sp., and *Escherichia coli*, respectively (Siehta *et al*., 1984). In addition, *Capsicum* traditionally been used in Indonesia to treat *Candida albicans* (Soetarno *et al*., 1997). Moreover, it has been demonstrated by Koffi-Nevry *et al*. (2012) the bactericidal effects of *Capsicum annuum* and *Capsicum frutescens.*

Acute and recurrent infections with herpes simplex virus type-1 (HSV-1) and type-2 (HSV-2) are widely spread worldwide, leading to a wide range of symptoms from weak to severe particularly in immunocompromised patients in whom the infection may become life threatening (Verma *et al*., 2008). Acyclovir is a nucleoside derivative drug has been widely used to cure HSVs infections (Vijayan *et al*., 2004). However, due to the high incidence of HSV infections and the widely use of this drug, new strains from the HSVs appear with high resistance to this class of treatment (Visintini Jaime *et al*., 2013). Thus, it is of highly importance to develop novel antiviral agents acting upon different targets to overcome the problem of viral resistance. Therefore, the current study was aimed to

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describe the antiviral activity of *Capsicum annuum* against HSV-1 and HSV-2 using Vero cells as an *in vitro* approach.

MATERIALS AND METHODS

Preparation of Capsicum annuum *methanolic extract (CaME)*

Briefly, the whole *Capsicum annuum* dried fruits were collected from a local market at Riyadh, Saudi Arabia during the months of March, 2016. The fruits were extracted with 70% methanol by shaking for 24 h at room temperature. The methanolic extract was then sieved using Whatman® grade 2 filter paper. After then, supernatant was evaporated under vacuum and dissolved in dimethyl sulfoxide (DMSO).

DPPH radical scavenging assay

Antiradical activity of *Capsicum annuum* methanolic extract was determined by using 1,1-diphenyl 1-2-picryl hydrazyl (DPPH) radical scavenging assay as described previously by Abdel Moneim (2013). A 2.5% DPPH was freshly made by dissolving 2.5 mg of DPPH in 100 ml of 100% methanol and an aliquot of 100 µl of the extract at the concentrations of 25, 50, 100, 150, 200 and 250 µg/ml were mixed with 3 ml of the DPPH solution, separately in clean tubes. Corresponding blank sample was prepared and ascorbic acid (1-100 µg/ml) was used as reference standard. The mixtures were incubated at room temperature in dark place for 20 min and the change in absorbance was read at 517 nm. The scavenging activity percentage of the sample was calculated by the equation, %DPPH scavenging = [(A control – A sample)/A control] -100, where A is the absorbance. Ascorbic acid was used as positive control and comparing with its' IC50.

Cell culture

Kidney epithelial cells extracted from an African green monkey (84113001/SIGMA**,** Vero Cell Line) were maintained in Dulbecco's modified Eagle's growth medium (DMEM, Invitrogen, Carlsbad, CA) with 10% FBS (fetal bovine serum, Invitrogen) and supplemented with 0.1% penicillin/0.1% streptomycin obtained from Invitrogen. Cell lines were maintained at 37°C, humidified atmosphere with 5% CO_2 according to the standard procedures and used for cytotoxicity and antivirus assays when approximately 80% confluent.

Cytotoxicity assay

Cell cytotoxicity was carried out using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay to check whether the methanol extract from *Capsicum annuum* at various concentrations showed toxicity to Vero cells. Vero cells were plated at an initial density of 1×10^4 cell/ml in a 96-well plate and treated with various concentrations of CaME (50–2500 µg/ml) or vehicle for 48 h. 0.5 mg/ml MTT (Sigma) was added to the cells were then incubated with at 37 °C for 4 h. The formed formazan crystals were extracted and dissolved using isopropanol. The absorbance after then was estimated with an ELISA reader at 562 nm. The resultant data were expressed as the percentage of viable cells relative to untreated controls.

Antiviral activity assay

80% confluent cell monolayers seeded in 96-well plates were incubated with increasing non-cytotoxic concentration of CaME, thus, 10, 25, 50, 100, 200 and 500 µg/ml of CaME were used. Six wells at least were used for every concentration. After 2 h, the cell were infected with HSV-1 or HSV-2 at a multiplicity of infection (MOI) of 0.001 pfu/cell, and re-incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. The viral cytopathic effect (CPE) was examined daily using a light microscope. When CPE was observed in all virus control wells, the 50% inhibitory concentration (IC_{50}) of viral CPE was estimated in comparison to the virus control, as described previously. Acyclovir (Sigma®) at concentration of 0.5, 1, 2, 4 and 5 µg/ml served as the positive control.

Viral plaque number reduction assay

Inhibition of HSV-1 and 2 replications were estimated with plaque reduction assay. Vero cells were distributed in 24-well plates at a density of 10×10^4 cells. The plaque reduction assay was done in infecting cell monolayers with HSV-1 or HSV-2 at a MOI of 0.001 pfu/cell in presence of serial dilutions of the non-cytotoxic concentration of CaME (10, 25, 50, 100, 200 and 500 µg/ml) for 2 h at 37°C in a humidified atmosphere of 5% CO_2 in air. The medium was subsequently discarded from the wells, and the cells were washed with clean medium twice and covered again with a fresh medium containing 1.2% methylcellulose (Sigma) with CaME at the same concentration. After incubation for 24 h at 37°C in humidified atmosphere with 5% CO_2, the supernatant was discarded, and the cells were fixed in 10% formalin in phosphate-buffered saline and then stained with 0.8% crystal violet in 50% ethanol and the viral plaques were counted microscopically at low power.

Statistical analysis

One-way ANOVA and statistical comparisons between the groups were performed with Duncan's test, using a statistical package program (SPSS version 17.0). Results represent the mean of three independent experiments ± standard deviations.

Table I.- Total polyphenolic content, total flavonoid content and antiradical activity of *Capsicum annuum* methanolic extract.

Extract	*Capsicum annuum* methanolic extract
Total polyphenolic[a]	76.3±4.36
Total flavonoid[b]	12.1±0.72
DPPH IC_{50}	64.1±3.2

[a], Total phenolic content in *Capsicum annuum* methanolic extract is determined as mg/g gallic acid equivalent of phenols/g dried extract; [b], Total flavonoid content in *Capsicum annuum* methanolic extract is determined as mg/g quercetin equivalents of flavonoids/g dried extract. Radical scavenging activity was measured changes in absorbance at 517 nm. Data are expressed as mean ± standard deviations of three independent experiments each performed in duplicate.

RESULTS AND DISCUSSION

As shown in Table I, the total polyphenolic content of *Capsicum annuum* methanolic extract was 76.3±4.36 mg/g gallic acid equivalent of phenols/g dried extract while, the total flavonoid content was 12.1±0.72 mg/g quercetin equivalents of flavonoids/g dried extract. *Capsicum annuum* is known as a good source of phytochemicals compounds such as polyphenols, carotenoids and ascorbic acid, and is recognized as beneficial to health. These phytochemicals display high antioxidant property, and their consumption has been linked to a decreased risk of developing chronic and degenerative diseases such as cancer and diabetes (Bode and Dong, 2011; Kwon *et al*., 2007). Among phytochemicals, polyphenols are particularly interesting for their free radical scavenging activities and *in vivo* biological activities. The major active compounds found in red pepper are capsaicinoids such as capsaicin, dihydrocapsaicin, and nordihydrocapsaicin. In accordance with our findings, Nascimento *et al*. (2014) found that the phenolic content ranged from 3.2 ± 0.22 to 110.6 ± 1.03 mg GAE g−1 of whole fruits of *C. frutescens* extract. On other hand, Yazdizadeh Shotorbani *et al*. (2013) found that the red pepper contains polyphenols ranged from 63 to 78 mg GAE g−1 of whole fruits of *C. annuum* extract. However, our finding in flavonoids is greater than that of recorded by Yazdizadeh Shotorbani *et al*. (2013) who reported the maximum flavonoids found in red pepper is 7.8 mg/g quercetin equivalents of flavonoids/g dried extract.

Furthermore, we determined the antioxidant activity of CaME by using DPPH radical scavenging activity assay. The highest antiradical activity based on the DPPH model system was found for CaME, which was comparable to the activity of vitamin C. The obtained data for the DPPH radical scavenging assay was shown in Table I. The data indicated that the 50% DPPH radical scavenging activity of CaME was 64.1 µg/ml while, the 50% DPPH radical scavenging activity of vitamin C was 68.5 µg/ml. The whole fruit of *Capsicum annuum methanolic extract* presented higher phenolics and flavonoids contents, and these contents could be correlated with the DPPH radical scavenging potential. The present finding was in agreement with Chavez-Mendoza *et al*. (2015), who reported that bell peppers contain high concentration of antioxidant activity. In this regard, Materska and Perucka (2005) attributed this activity to capsaicin and dihydrocapsaicin found in the fruit. The antioxidant properties of phenolic compounds originate from their properties of proton loss, chelate formation, and dismutation of radicals. Phenols are compounds that have the ability to destroy radicals because they contain hydroxyl groups.

In addition, the cytotoxicity assay was performed using MTT to assess the metabolically active cells which convert the dye to a water-insoluble dark blue formazan by reduction process of splitting tetrazolium ring (Alvarez *et al*., 2015). The cytotoxicity of CaME against Vero cells is summarized and shown in Figure 1. The CC_{50} value of CaME against Vero cells was 1078.69 µg/ml. According to our findings, CaME has low cytotoxicity and Vero cells can tolerate it well. The low toxicity of CaME was reported by Viktorija *et al*. (2013) who noted that capsicum extract did not show any cytotoxic effect at the examined concentrations while, capsaicin alone (100 µM) showed high cytotoxicity and they assumed that the other compounds (carotenoids, vitamins, and other polyphenolic substances) within the extract interact antagonistic with the cytotoxic effect of capsaicin. However, Park *et al*. (2012)

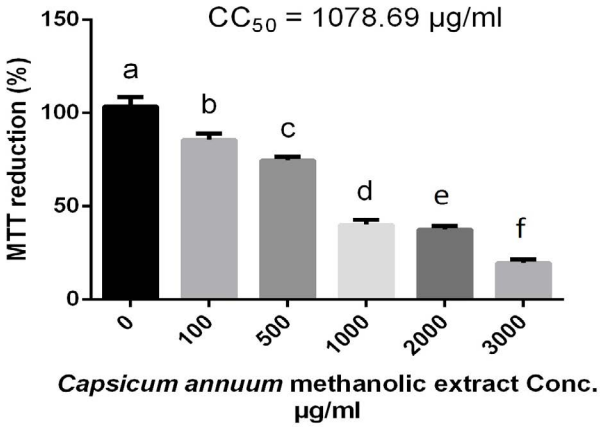

Fig. 1. The cytotoxic effect of *Capsicum annuum* methanolic extract. CC_{50} is the concentration of the 50% cytotoxic effect. Data are showed as Mean of three independent experiments each performed in duplicate. Means indicated with different letters differ significantly.

Table II.- The antiviral activity of *Capsicum annuum* methanolic extract.

CaME	Cell Cytopathic Effect (CPE)	
	HSV-1	HSV-2
10 µg/ml	-	-
25 µg/ml	-/+	-
50 µg/ml	+	-/+
100 µg/ml	+	+
200 µg/ml	+	+
500 µg/ml	+	+

+, Virus inhibition; +/-, Partial inhibition; -, No inhibition, presence.

found that the proliferation of Vero cells was significantly reduced by CaME and they attributed the cytotoxicity of red pepper to its antioxidant activity.

Results of the antiviral activity and the cytopathic inhibitory assay of CaME against HSV-1 and HSV-2 were presented in Table II. Concentrations from 50-500 µg/ml completely inhibit *in vitro* growth of HSV-1 with a partial inhibition at 25 µg/ml, while concentrations from 100-500 µg/ml completely inhibit *in vitro* growth of HSV-2 with a partial inhibition at 50 µg/ml. The present findings were in accordance with Ozcelik *et al*. (2011) and Carole *et al*. (2015). However, Bourne *et al*. (1999) stated that capsaicin does not have a direct anti-herpes effect, whereas civamide (cis-capsaicin) exhibited remarkable inhibition against genital HSV.

As illustrated in Figure 2, CaME at 40 µg/ml showed 50% inhibition of plaque of HSV-1 and provided 100% inhibition against HSV-1 at 200-500 µg/ml as indicated by the plaque inhibition assay. Likewise, the anti-HSV-2 potential of the CaME was performed and the results were illustrated in Figure 3. The HSV-2 was more tolerate to CaME and according to the obtained results, CaME at 40 µg/ml provided 40% inhibition of plaque of HSV-2 and provided 100% inhibition against HSV-2 at 200 - 500 µg/ml. The present findings might be indicating the anti-herpes simplex virus types-1 and 2 of *Capsicum annuum* and these activities due to the ability of phytochemical compounds in the extract to interfere at the attachment points and penetration of the virus in cells. The principal act of the extract is probably modified the cellular and viral receptors, preventing the virus from attaching to cells. According to Obi *et al*. (2006), if the receiving cells are modified before the viral infection, the virus's ability to bind and penetrate the cells would be greatly reduced. Indeed, the study of Kouassi and Koffi-Nevry (2012) showed that *Capsicum annuum* contains biological active compounds and vitamins such as ascorbic acid and the ß-carotene are responsible for these activities.

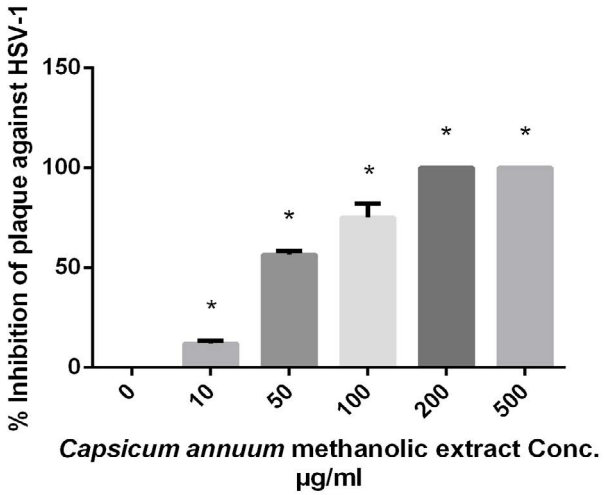

Fig. 2. Inhibition activity of *Capsicum annuum* methanolic extract against HSV-1. IC_{50} is the concentration of the extract required to inhibit 50% virus-induced cytopathic effect. Results are represented as Mean of two independent experiments each performed in triplicate. Means indicated with (*) differ significantly.

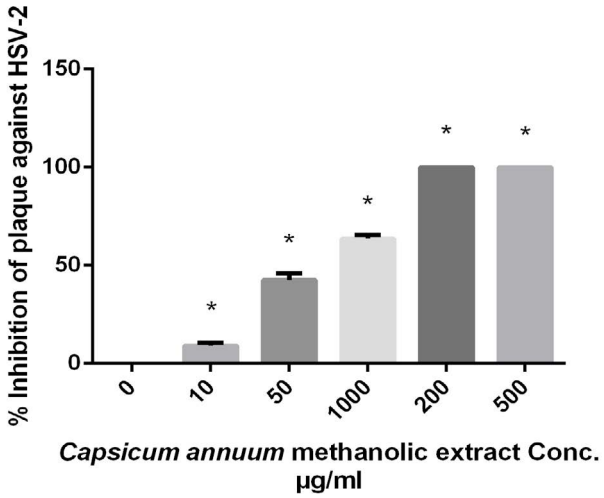

Fig. 3. Inhibition activity of *Capsicum annuum* methanolic extract against HSV-2. IC_{50} is the concentration of the extract required to inhibit 50% virus-induced cytopathic effect. Results are represented as Mean of two independent experiments each performed in triplicate. Means indicated with (*) differ significantly.

CONCLUSION

In conclusion, the crude extract of *Capsicum annuum*

was active against HSV-1 and HSV-2 and demonstrated antiviral activity with low cytotoxicity which may be used in combination with standard drug to treat herpes infection.

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Conflict of interest statement

We declare that we have no conflict of interest.

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